REMARKS

These remarks are in response to the Final Office Action mailed November 25, 2008. No claims have been amended and thus a further search is not required. No new matter is believed to have been introduced.

Applicants respectfully thank Examiner Popa and Supervisor Woitach for meeting with Applicant and Applicants' representatives Dr. Jolly and Joseph Baker. At the interview Applicants explained the novelty and inventiveness of the Applicants' technology and the unexpected results achieved including increased stability through multiple passages and the transformation and spread through neoplastic cells and tissues. The parties did not reach and agreement; however, the Examiner and the Examiner's supervisor indicated that they would consider additional evidence indicating the why one would not combine the references as suggested in the Office Action as well as evidence of teaching away. Applicants have provided such evidence here and can provide additional evidence should the Examiner wish to consider additional information.

I. NON-STATUTORY OBVIOUSNESS-TYPE DOUBLE PATENTING

Claims 41, 43-45, 49-51, 56, 58, 59, 61, 63-73, 75, 78-82 and 87-121 stand provisional rejected on the grounds of non-statutory obviousness-type double patenting as allegedly being unpatentable over claims 22, 23, and 26-34 of co-pending application no. 11/805,411 (the '411 application) in view of both Yan *et al.* (Prostrate, 32:129-139, 1997) and Sobol *et al.* (U.S. Patent No. 5,674,486). Applicants respectfully traverse this rejection.

Applicants respectfully submit that because there can be no time-wise extension of the patent term in the present application beyond that of the '411 that a Terminal Disclaimer would only be suitable in the '411 application since (1) the '411 was the later filed application and (2) because the patent term would be properly adjusted to coincide with the patent term of the present application. The public would not be served by a Terminal Disclaimer in the present application. Furthermore, because obviousness-type double patenting is claim dependent and not disclosure dependent and because no allowable subject matter has been identified in either the present application or the '411, Applicants respectfully request

that the rejection be held in abeyance until such time as allowable subject matter is identified.

II. REJECTION UNDER 35 U.S.C. §103

Claims 41, 43-45, 49-51, 56, 61, 66, 70, 71, 75, 78-80, 87, 89, 91, 97-102, 105, 107, 109, 115-119, and 121 stands rejected under 35 U.S.C. §103 as allegedly unpatentable over Ram *et al.* (Cancer Research, 1993, 53:83-88) in view of each of Martuza et a. (U.S. Patent No. 5,585,096), Murakami *et al.*, (Gene, 1997, 202:23-29) and Sobol *et al.* (U.S. Patent No. 5,674,486).

Claims 41, 43-45, 49-51, 56, 61, 66, 70, 71, 75, 78-80, 87, 89, 91, 97-102, 105, 107, 109, 115-121 are rejected as allegedly unpatentable over Ram *et al.* (Cancer Research, 1993, 53:83-88) in view of each of Martuza et a. (U.S. Patent No. 5,585,096), Murakami *et al.*, (Gene, 1997, 202:23-29) and Sobol *et al.* (U.S. Patent No. 5,674,486) as above and further in view of Douar *et al.* (Gene Ther, 3:780-796, 1996), which is further combined to overcome certain deficiencies in the primary set of references.

Claims 41, 43-45, 49-51, 56, 58, 59, 61, 66, 70, 71, 73, 75, 78-80, 87-92, 97-102, 105-110, 115-119, and 121 are rejected as allegedly unpatentable over Ram *et al.* (Cancer Research, 1993, 53:83-88) in view of each of Martuza et a. (U.S. Patent No. 5,585,096), Murakami *et al.*, (Gene, 1997, 202:23-29) and Sobol *et al.* (U.S. Patent No. 5,674,486) as above and further combined with Vile *et al.* (Virology, 214:307-313, 1995) and Yan *et al.* (Prostrate, 32:129-139, 1997), which are further combined to overcome certain deficiencies in the primary set of references.

Claims 41, 43-45, 49-51, 56, 58, 61, 63-73, 75, 78-82, 87-119, and 121 are rejected as allegedly unpatentable over Ram *et al.* (Cancer Research, 1993, 53:83-88) in view of each of Martuza et a. (U.S. Patent No. 5,585,096), Murakami *et al.*, (Gene, 1997, 202:23-29) and Sobol *et al.* (U.S. Patent No. 5,674,486) as above, and further combined with Vile *et al.* (Virology, 214:307-313, 1995) and Kasahara *et al.* (Science, 266:1373-1376, 1994), to overcome certain deficiencies in the primary set of references.

Applicants respectfully traverse these rejections as set forth previously in the Response filed August 2008, which is incorporated herein by reference. Furthermore, Applicants provide the following additional remarks and information.

Ram *et al.* provides replication defective retroviral vectors that require a helper cell line for replication. Martuza *et al.* provides a DNA viral vector derived from Herpes Simplex Virus, a complex lytic DNA virus that induces cytotoxicity. Murakami *et al.* provides recombinant Rous Sarcoma Virus that cannot infect mammalian cells and which comprises a disposable gene thus provided a flexible naturally inherent cassette.

Turning to the three references of Ram *et al.*, Martuza *et al.* and Murakami *et al.*, Applicants respectfully submit that one of skill in the art would not be motivated to generate Applicants' claimed invention from any combination of the foregoing references. It is important to understand that the uses, genomes and ability to infect mammalian cells are drastically different in each of the vector systems described in these references. It is not a matter of piecing together the various references as suggested by the Office Action. For example, truly piecing the references together would require placing a DNA genome in an RNA vector that can only infect avian cells and somehow making it infect mammalian cells without losing stability. As described in the prior response, one of skill in the art would not mix and match the genomes of the various vectors as suggested by the Office Action.

For example, the Examiner is directed to Exhibit A (Oh, *et al.*, J. of Virol., 76(4):1762-1768, 2002), which demonstrates that even after the priority date of the present invention, those of skill in the art would not have been motivated to generate vectors as set forth by the present claims by mixing/matching RSV and oncoretroviruses. For example, paragraph 2, column 1 at page 1762 of Oh *et al.*, states:

> Most retroviral genomes cannot accommodate the insertion of significant amounts of additional genetic information. In these cases, viral sequences must be removed to provide a place for whatever additional information is inserted. Such viruses are, by definition, replication defective. The missing viral genetic information must be supplied in *trans*, either by a helper cell or a helper virus. *There is one exception*. Avian leukosis viruses can accept approximately 2.5 kb of additional information: the naturally occurring avian leukosis virus derivative Rous sarcoma virus (RSV)

> contains, in addition to a full complement of viral genes, the *src* oncogene (21).
>
> (emphasis ours).

Simply put, the foregoing paragraph actually teaches away from the use of mammalian oncoretroviral vectors because they must be rendered "defective" to accommodate additional genetic material, unless you use the "one exception" an RSV vector which includes the dispensible *src* gene. This statement and the cited reference are consistent with the remarks Applicants provided in the prior response – simply that one of skill in the art would not modify the teachings of Murakami *et al.* (regarding RSV viral vectors) to non-RSV vector systems due to the inability of such mammalian retroviral systems to accommodate additional genetic sequences.

The present application describes compositions and methods whereby this "inability" is overcome. Such an advance in the art is one of the main purposes and policies behind the patent system, i.e., to protect advances in the art and give to the inventors a limited time of exclusivity for their hard work and development.

Furthermore, turning to Martuza *et al.*, one of skill in the art would recognize that the genome of Herpes virus has little if any similarity in its genome, infectivity or life cycle to oncoretroviral vectors such as MLV. For example, Herpes virus is a lytic virus having cellular toxicity. In addition, herpes virus is far more complex and is a DNA virus. One of skill in the art would not translate the teachings in the Herpes viral arts to those of the oncoretroviral arts due to such drastic differences in genomes, infectivity and viral life cycle.

Thus, the three references of Ram *et al.*, Martuza *et al.*, and Murakami *et al.* cannot be combined without substantial changes to the genomes of the vectors described and modifications of the references that are not suggested by the art. In other words, one of skill in the art would have to discard the teachings of the references themselves to arrive at Applicants' claimed invention. For example, in order to utilize Sobol *et al.* as suggested by the Office Action, one of skill in the art would have to discard the teaching in the reference that replication competent vectors should be discarded as being dangerous. As another example, one of skill in the art would have to discard the teachings of Oh *et al.* and others in the art that

oncoretroviral vectors can be modified to incorporate additional sequences even though Oh *et al.* says RSV is the "one exception".

Applicants through extensive experimentation and development demonstrate that not just any combination of elements (as suggested by the Office Action), not just any insertion site (as suggested by the Office Action) and not just any viral vector (as suggested by the Office Action) would result in Applicants' claimed invention. Applicants were the first to discover that the combination of virus selection and IRES cassette insertion site provides a competent, stable and effective RCR system for treating cell proliferative disorders. For example, the Examiner is respectfully directed to Logg *et al.*, J. of Virol., 75(15):6989-6998, 2001, which sets forth the importance of the cassette location. The combination of transduction efficiency, transgene stability and target selectivity was unknown in any recombinant replication competent mammalian oncoretrovirus prior the instant vector. The methods (and the vector composition used in the methods) provides insert stability and maintains transcription activity of the transgene and the translational viability of the encoded polypeptide.

Applicants respectfully submit that the high-level view taken by the Office (1) fails to consider the modifications that must be made to each reference to arrive at the invention that go far beyond mere routine experimentation and (2) disregard accepted principles at the time the invention was made that replication competent retroviral vectors were unacceptable, that RSV was an "exception" to the viral vector limitations, and that HSV are not lytic and can be modified for the same purpose as the present invention. Applicants respectfully submit that the foregoing (i) lack of motivation, (ii) modifications to the references required to make them even remotely functional, and (iii) general disregard for accept principles at the time of the invention, cannot be merely discarded by a general assertion to the skill in the art.

To again put the combination of the cited references in context and to demonstrate the lack of a *prima facie* case of obviousness each reference must be explained for what it describes and then and only then can it be analyzed in combination. As will become apparent from the following remarks, the references either individually or in combination do not provide the necessary factors to set forth a *prima facie* case of obviousness.

As the Examiner indicates Ram *et al.* fail to teach or suggest a recombinant replication competent oncoretroviral vector or recombinant plasmid or recombinant polynucleotide encoding a replication competent oncoretroviral vector. Furthermore, Ram *et al.* fail to teach or suggest treating a tumor in the absence of a helper cell to assist in the defective viral replication, Ram *et al.* fails to teach or suggest a cytokine transgene, Ram *et al.* fails to teach or suggest a chimeric env protein, Ram *et al.* fails to teach or suggest a tissue specific promoter, and Ram *et al.* fails to teach or suggest an IRES cassette. Ram *et al.* is far removed from the methods and compositions of Applicants' invention, which utilize a replication competent, non-helper cell system to treat a cell proliferative disease or disorder.

The cited reference of Ram *et al.* describes a method that utilizes "retroviral producer cells" injected at the site of a tumor (see page 86, column 2, last paragraph of the cited reference). The producer cells support the *in situ* production of a retroviral vector containing a suicide gene. The producer cells are necessary because the vector is not replication competent. Further, the nucleic acid sequence encoding the suicide gene is located "just downstream of the 5' long terminal repeat sequence" (see page 84, column 1, lines 2-4 of the cited reference) in a location different from Applicants' claimed vector. It is clear from the contents of the cited reference that Ram *et al.* fail to appreciate the significance of utilizing a replication competent oncoretrovirus in the absence of a producer cell to achieve efficient transduction. Because Ram *et al.* use a gutted vector in order to incorporate the transgenes (see, Oh *et al.* described above), transcription of a transgene can easily be effected off the regulatory region of the 5'LTR. In contrast, Applicants' transgene is not directly linked to the 5'LTR. The location of the transgene and the IRES as set forth in Applicants' claims is not an insignificant modification.

Thus, Ram *et al.* is deficient in at least three aspects: (1) the vector is replication defective; (2) the methods require a help cell; and (3) the transgene location is of little or no important to Ram *et al.* The gutted size and location of the transgene in Ram *et al.* allow for the 5' LTR to serve as the regulatory region. To overcome these deficiencies the Office combines Ram *et al.* with Martuza *et al.*

The cited reference of Martuza *et al.* allegedly teach replication competent viral vectors derived from adenovirus and herpes simplex virus (such vectors are

DNA vectors - very different than RNA vectors). Applicants respectfully submit this is the first of many leaps the Office makes to overcome voids in the development of Applicants' claimed invention. First, it is not clear why one would combine a defective retrovirus of Ram *et al.* with a DNA virus of Martuza *et al.*, the genomes are completely different. Nevertheless, when the references are combined the combination still fails to teach or suggest Applicants' claimed invention. Like Ram *et al.*, Martuza *et al.* fail to appreciate the importance of positioning a heterologous sequence encoding a therapeutic polypeptide in a region outside the LTR or not linked directly to the LTR of the viral vector. Nor does the combination of references teach, suggest, or appreciate an internal ribosome entry site. As will be recognized by the Examiner and those of skill in the art, merely inserting a transgene into a replication competent retrovirus does not provide a reasonable expectation that infectivity, stability or continued transmission and expression of the transgene will occur. In fact, numerous peer-reviewed journal articles indicate that insertion of transgene into U3 and other locations within a replication competent retrovirus can cause a loss of replication, and genetic instability of the vector (see, e.g., Logg *et al. supra*). Thus, the combination of Ram *et al.* and Martuza *et al.* fail to teach or suggest Applicants' claimed invention and do not provide any reasonable expectation of success in achieving a RCR having the transmission and genetic stability of Applicants' claimed vectors.

To overcome the deficiencies of Ram *et al.* and Martuza *et al.*, the Office combines Murakami *et al.*

Murakami *et al.* use a Rous Sarcoma Virus. As stated by Oh *et al.* (Exhibit A), the RSV is the one "exception" to retroviruses. The IRES-transgene insertions described in Murakami *et al.* consist of an IRES-transgene sequence positioned 3' to the env-encoding sequence and 5' to the 3' LTR. However, the cited reference utilizes replication competent avian sarcoma viruses (RCAS) which are distinct from the oncoretroviruses of the pending claims and incapable of replication in mammalian cells. Thus, the RSV vector could not be used to treat a mammal as set forth in Applicants' claims. The inability of RSV to produce infective viral particles in mammalian cells is disclosed in several peer-reviewed journal articles. Here, again, the Office makes a leap from a defective gutted retroviruses, to DNA viruses to avian

viruses, with little direction, suggestion or likelihood of success in the art particularly when the RSV virus is recognized as an exception for its ability to incorporate transgenes into its genome. It is only through Applicants' disclosure and hindsight reconstruction that such very different viral architectures and functions can be pieced together. For example, Avian Rous Sarcoma Virus naturally carries extra sequences (the src oncogene, which is in addition to the gag, pol and env genes required for replication, and which is similar in size to the env gene) positioned just after env. Thus, RSV evolved a capacity to incorporate a large piece of extra sequence in this location in its genome, something not found in mammalian oncoretroviruses. The idea of putting an IRES-transgene insert after the *env* gene in a mammalian oncoretrovirus would not be obvious in view of the cited references simply because there are no known naturally-evolved replication-competent mammalian oncoretroviruses with extra genes following the *env* (or anywhere else, for that matter). In fact, it was recognized in the art that inserting a transgene in the region following the env gene although providing short term expression ultimately resulted in genetic instability and loss of the transgene in subsequent rounds of replication. RSV through natural development has developed a "transgene insertion site" because it contained a non-essential and replaceable gene (*src*), thus providing additional flexibility (i.e., an "exception") compared to mammalian oncoretroviruses.

Further, combining the IRES-transgene of Murakami *et al.* and the vector described by Martuza *et al.* would not result in a vector or method described or claimed in the instant application. It is not clear why or how one would combine a DNA viral vector and an RNA avian viral vector.

Finally, the Office combines Sobol *et al.* with Ram *et al.*, Martuza *et al.*, and Murakami *et al.*, for the teaching of cytokines to treat cancer. The Office appears to be picking and choosing the use of certain reference and avoiding the teachings of the reference as a whole. When taken as a whole, Sobol *et al.* actually teach that one should avoid the use of replication competent retroviruses. For examples, Sobol *et al.* teach throughout the specification that one should use proper screening, production and removal of replication competent retroviruses from any system or method. However, even in view of such a teaching away, Sobol *et al.* do not remedy

the deficiencies as set forth above regarding the replication competent retrovirus and use of such recombinant viral vectors for the treatment of cell proliferative disorder.

Not only do the cited references when combined fail to identify predictable solutions for achieving a replication competent oncoretrovirus capable of delivering a therapeutic polypeptide to dividing cells, they also fail to provide all the components necessary for the production of the vector set forth in the claimed methods.

In contrast, the Applicants have succeeded in developing a replication competent oncoretroviral vector with an enhanced capability to stably deliver a heterologous sequence to a dividing mammalian cell. Once integrated into a target cell, the novel vector produces a therapeutic polypeptide encoded by the heterologous sequence. In addition, viral particles which infect neighboring dividing cells are also produced in the absence of helper cells.

It is important to understand that the surprising combination of transduction efficiency, transgene stability, and target selectivity provided by Applicants' inventions were simply unknown in any recombinant replication competent mammalian oncoretrovirus prior to the Applicants' invention. When placed in a mammalian oncoretroviral background, the cassette is useful for the stable expression of a transgene coding sequences including marker genes such as green fluorescent protein (GFP), suicide genes such as thymidine kinase, cytosine deaminase (CD) or purine nucleoside phosphorylase (PNP), and genes encoding cytokines such as interferon.

For at least the foregoing reasons, the pending claims are novel and non-obvious over the cited reference. Accordingly, Applicants respectfully request withdrawal of this rejection.

III. REJECTION UNDER 35 U.S.C. §112, FIRST PARAGRAPH

Claims 41, 43-45, 49-51, 56, 58, 59, 61, 63-73, 75, 78-82, and 87-121 stand rejected under 35 U.S.C. §112, first paragraph as allegedly failing to comply with the written description requirement. In particular, the Office Action alleges that the term, "at the 5' or 3' end" is new matter. Applicants respectfully traverse this rejection.

As set forth previously, Applicants respectfully direct the Examiner to page 64, lines 16-20, which explains that the LTR need only be present at one or both of the

5' or 3' ends and that during reverse transcription the LTR is duplicated. Accordingly, delivery of a vector comprising a single LTR would be sufficiently duplicated during the RCR life cycle (see also page 67 and figure 8). Furthermore, page 18 and 19 explain the viral life cycle including that upon transcription the provirus "now" has two identical repeats at either end. The use of "now" indicates that it did not previously have LTRs at both ends but only after transcription of the provirus does it "now" have LTRs at both ends.

However, to advance prosecution and without disclaimer, and in order to place the case in better form for appeal, Applicants have amended the claims to remove "5' or 3'".

For at least the foregoing, the Applicant submits that the claimed invention is patentable and request reconsideration and notice of such allowable subject matter.

The Director is authorized to charge any required fee or credit any overpayment to Deposit Account Number 50-4586, please reference the attorney docket number above.

The Examiner is invited to contact the undersigned at the below-listed telephone number, if it is believed that prosecution of this application may be assisted thereby.

Respectfully submitted,

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EXHIBIT A

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Construction and Characterization of a Replication-Competent Retroviral Shuttle Vector Plasmid

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We constructed two versions of an RCASBP-based retroviral shuttle vector, RSVP (RCASBP shuttle vector plasmid), containing either the zeocin or blasticidin resistance gene. In this vector, the drug resistance gene is expressed in avian cells from the long terminal repeat (LTR) promoter, whereas in bacteria the resistance gene is expressed from a bacterial promoter. The vector contains a bacterial origin of replication (ColE1) to allow circular viral DNA to replicate as a plasmid in bacteria. The vector also contains the *lac* operator sequence, which binds to the *lac* repressor protein, providing a simple and rapid way to purify the vector DNA. The RSVP plasmid contains the following sequence starting with the 5′ end: LTR, *gag*, *pol*, *env*, drug resistance gene, *lac* operator, ColE1, LTR. After this plasmid was transfected into DF-1 cells, we were able to rescue the circularized unintegrated viral DNA from RSVP simply by transforming the Hirt DNA into *Escherichia coli*. Furthermore, we were able to rescue the integrated provirus. DNA from infected cells was digested with an appropriate restriction enzyme (*Cla*I) and the vector-containing segments were enriched using *lac* repressor protein and then self-ligated. These enriched fractions were used to transform *E. coli*. The transformation was successful and we did recover integration sites, but higher-efficiency rescue was obtained with electroporation. The vector is relatively stable upon passage in avian cells. Southern blot analyses of genomic DNAs derived from successive viral passages under nonselective conditions showed that the cassette (drug resistance gene-*lac* operator-ColE1) insert was present in the vector up to the third viral passage for both resistance genes, which suggests that the RSVP vectors are stable for approximately three viral passages. Together, these results showed that RSVP vectors are useful tools for cloning unintegrated or integrated viral DNAs.

The retroviral life cycle depends on the conversion of the RNA genome found in virions into DNA and the subsequent integration of the DNA into the host cell genome. There are a number of different types of experiments for which the molecular cloning of either unintegrated or integrated viral DNA is a critical step. In most cases, cloning the viral DNA involves standard recombinant DNA techniques: viral DNA is inserted into a plasmid or a prokaryotic viral vector. However, there is an alternative approach. It is possible to introduce elements into retroviral vectors that allow DNA forms of the viral genome to replicate in prokaryotic hosts. These types of vectors are referred to as shuttle vectors and can simplify the recovery of viral DNA. At a minimum the shuttle vector must have a plasmid origin of replication and a gene (promoter and coding region) that can be selected in a bacteria.

Most retroviral genomes cannot accommodate the insertion of significant amounts of additional genetic information. In these cases, viral sequences must be removed to provide a place for whatever additional information is inserted. Such viruses are, by definition, replication defective. The missing viral genetic information must be supplied in *trans*, either by a helper cell or a helper virus. There is one exception. Avian leukosis viruses can accept approximately 2.5 kb of additional information: the naturally occurring avian leukosis virus derivative Rous sarcoma virus (RSV) contains, in addition to a full complement of viral genes, the *src* oncogene (21). We have prepared a family of replication-competent retroviral vectors, collectively called the RCAS vectors, that derive from the Schmidt-Ruppin strain of RSV. Basically, in the RCAS vectors, the *src* gene has been deleted and a unique restriction site has been left at the site of the deletion to simplify the insertion of foreign DNA. To permit the cloning and amplification of the RCAS vector DNA, the viral genome was linked to pBR322-derived plasmid sequences (10); however, the plasmid sequences lie outside the viral replicon and the viral vector brings none of the prokaryotic plasmid sequences with it when it replicates in avian cells. It is possible to insert prokaryotic plasmid sequences into the *Cla*I site of an RCAS vector. The following two versions have been created: p779NC327AC28F, which contains a pBR origin and an ampicillin resistance gene (unpublished observations), and a derivative, pANV-A, which contains a pBR origin, a simian virus 40 promoter, and a neomycin resistance gene linked to the Tn*5* promoter (15).

The p779NC327AC28F plasmid has two disadvantages. First, there is no selection possible when the virus is propagated in avian cells. Second, the prokaryotic sequences are rapidly lost during virus propagation (unpublished observations). pANV-A can be selected in both prokaryotic and eukaryotic hosts, and the viral genome was reported to be stable through one round of viral replication (15).

We have revisited the RCAS shuttle vector problem and have prepared two new vectors that have advantages over the published vectors. First, the new vectors make use of drug resistance markers that are small (zeocin resistance and blasticidin resistance). Zeocin is a member of the bleomycin/phleomycin family of antibiotics and is a basic, water-soluble, copper-chelated glycopeptide isolated from *Streptomyces verticillus*

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(2). When zeocin enters the cell, the copper cation is reduced from Cu^{2+} to Cu^{+} and zeocin becomes activated. Zeocin will then bind DNA and cleave it, causing cell death (2). A zeocin resistance protein, the product of the *Streptoalloteichus hindustanus ble* gene, has been isolated from *S. hindustanus*, binds to zeocin, and inhibits its DNA cleavage activity (4, 6). The *S. hindustanus ble* gene has been used as a selectable marker for prokaryotes and eukaryotes (3–5, 14). Blasticidin S is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* that is a potent protein synthesis inhibitor for both prokaryotes and eukaryotes (24, 25). Blasticidin resistance is conferred by the expression of one of two blasticidin S deaminase genes, either the *BSD* gene of *Aspergillus terreus* (12) or *bsr* of *Bacillus cereus* (11). These enzymes convert blasticidin S to an inactive deaminohydroxy derivative (11). In eukaryotic cells, the RSVP vectors express the drug resistance genes via a spliced message, so that an internal promoter is not needed. In addition, we have added a completely symmetric *lac* operator, which makes it a simple matter to enrich for viral DNA (16, 17). This symmetric *lac* operator, which is an inverted repeat of a 15-bp segment from the left half of the natural operator sequence (5′-TGTGGAATTGTGAGCGCTCACAATTCCACA-3′), has been reported to bind the *lac* repressor 10-fold more tightly than the natural *lac* operator sequence (19). It is possible with this system not only to recover unintegrated circular viral DNA from infected cells but also to easily clone integration sites. The blasticidin and zeocin cassettes are approximately 1.2 kb long, so it should be possible to introduce additional information into the shuttle vectors. We have also demonstrated that the RSVP vectors are sufficiently stable that they can be passaged three times without a substantial loss of the prokaryotic plasmid sequences.

MATERIALS AND METHODS

Plasmid construction. A cassette containing multiple cloning sites (MCS), a drug resistance gene (either zeocin or blasticidin), a *lac* operator sequence, and a ColE1 replication origin was introduced into the plasmid 327AC (10) as described below. The plasmid 327AC was digested with *Eco*RI and *Hin*dIII, and the digested DNA was ligated with two complementary 40-bp oligonucleotides that included the following cloning sites: *Cla*I, *Bgl*II, *Avr*II, *Not*I, *Xba*I, and *Hin*dIII. The resulting plasmid was called 327ACMC. The blasticidin resistance gene was PCR amplified from the pcDNA6/V5-His/*lac*Z plasmid (InVitrogen, Carlsbad, Calif.) by using the forward primer Bsd-Not, which anneals to the region upstream of the EM-7 promoter, and the reverse primer Bsd-Xba, which spans the termination codon for the blasticidin resistance gene. The sequence of Bsd-Not was 5′-ATCAgcggccgcATCAGCACGTGTTGAC-3′. The Bsd-Not primer contains a *Not*I restriction site in the overhang, which is indicated by lowercase letters. The sequence of Bsd-Xba was 5′-ACtctagaTTAGCCCTC CCACACATAACC-3′. The *Xba*I restriction site is indicated by lowercase letters, and the stop codon is underlined. The PCR product was cleaved with *Not*I and *Xba*I and inserted into the vector 327ACMC that had been digested with *Not*I and *Xba*I, which generated 327(Bsd). Next, two cDNA oligonucleotides (LacO-Xba, CTAGA**TGTGGAATTGTGAGCGCTCACAATTCCACA**ggatccTA; LacO-Hind, AGCTTAAggatcc**TGTGGAATTGTGAGCGCTCACAATTCCACA** T), containing the *lac* operator (bold) and a new *Bam*HI site (lowercase), were annealed and inserted into the *Xba*I/*Hin*dIII site of 327(Bsd), which generated 327(Bsd/Lac). The *lac* operator was sequenced to check its integrity. To create unique MCS, two cDNA oligonucleotides (MCS-Cla, CGATACTAGTCGTAC GATGCATGC; MCS-Not, GGCCGCATGCATCGTACGACTAGTAT), containing *Spe*I, *Spl*I, and *Nsi*I sites, were annealed and inserted into the *Cla*I/*Not*I site of 327(Bsd/Lac), which generated 327(MCS/Bsd/Lac). The ColE1 origin in the cassette was PCR amplified from the pcDNA6/V5-His/*lac*Z plasmid (InVitrogen) using the forward primer Ori-*Bam*H (TTCGGATCCATGTGAGCA AAAGGCCAGCAA) and the reverse primer Ori-Hind (GTCAAGCTTacgcgt CCCGTAGAAA AGATCAAAGGA). The reverse primer created an *Mlu*I site (lowercase letters) which was used in subsequent cloning steps. The PCR product was cleaved with *Bam*HI and *Hin*dIII and inserted into the *Bam*HI/*Hin*dIII site of 327(MCS/Bsd/Lac). The resulting plasmid was called 327ca(Bsd).

The zeocin resistance gene with the associated EM-7 promoter was PCR amplified from the pZeoSV2(+) plasmid (InVitrogen) using two primers, Zeo-Not (ATTgcggccgcTGTTGACAATTAATCATCGGC) and Zeo-Xba (GCCtcta gaTCAGTCCTGCTCCTCGGCCAC). A *Not*I site (lowercase) in the Zeo-Not primer was introduced upstream of the EM promoter and an *Xba*I site (lowercase) in the Zeo-Xba primer was introduced immediately downstream of the stop codon (underlined). The resulting PCR fragment was digested with *Not*I and *Xba*I and used to replace the *Not*I/*Xba*I fragment containing the blasticidin resistance gene of 327ca(Bsd), resulting in the plasmid 327ca(Zeo).

The retroviral backbone was constructed in the plasmid 779/2795 (9). To insert the *pol* region from the Bryan strain of RSV, a 4.7-kb *Sac*I-to-*Kpn*I fragment containing the *gag-pol* region of 779/2795 was replaced with a *Sac*I-to-*Kpn*I fragment of RCASBP(A) (18), which generated the plasmid 779BP. The cassette described above was purified as a *Cla*I-to-*Mlu*I fragment from 327ca(Bsd) and 327ca(Zeo) and was inserted into the *Cla*I-to-*Mlu*I site of 779BP to generate 779BP-ca(Bsd) and 779BP-ca(Zeo), respectively. These vectors, however, contained an ampicillin resistance gene and a second replication origin derived from pBR322 that was present in the original 779/2795 plasmid. The *Sal*I fragment containing both the ampicillin gene and the second origin was removed from the 779BP-ca(Bsd) and 779BP-ca(Zeo) plasmids to generate 779(ΔSal)BP-ca(Bsd) and 779(ΔSal)BP-ca(Zeo) plasmids. Finally, to introduce a 3′ splice acceptor site upstream from the drug-resistant genes in these vectors, the splice acceptor-containing segment was taken from RCASBP(A) as a *Sal*I-*Cla*I fragment. This fragment was used to replace the corresponding *Sal*I-*Cla*I segment in 779(ΔSal) BP-ca(Bsd) and 779(ΔSal)BP-ca(Zeo), which generated RSVP(A)B and RSVP (A)Z, respectively.

Cells, transfection, and infection. DF-1, a continuous line of chicken fibroblasts, was derived from EV-O embryos (7, 20). The cells were maintained in Dulbecco's modified Eagle medium (GIBCO BRL, Gaithersburg, Md.) supplemented with 5% fetal bovine serum, 5% newborn calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin (Quality Biological, Inc., Gaithersburg, Md.) per ml and incubated at 39°C with 5% CO_2. Cells were passaged 1:3 at confluence with trypsin DeLarco (pH 6.8). Plasmid DNA was introduced into DF-1 cells by calcium phosphate precipitation (13). Precipitates containing 10 μg of DNA per 100-mm-diameter plate were incubated with subconfluent DF-1 cells for 4 h at 39°C and then with medium containing 15% glycerol for 5 min at 39°C. Cells were washed twice with phosphate-buffered saline and incubated in growth medium for 48 h. The transfected cells were passaged two to three times to let the viruses spread throughout the culture. Culture medium containing the virus was harvested and subjected to low-speed centrifugation to remove cellular debris. A portion of the infectious virions was used to infect fresh DF-1 cells. Selection for drug resistance was initiated at 48 h postinfection at 10 μg/ml for blasticidin (InVitrogen) and 400 μg/ml for zeocin (InVitrogen).

Cloning unintegrated viral DNA into *Escherichia coli*. The circularized unintegrated viral DNA was recovered from infected cells by the method of Hirt (8). Three hundred nanograms of the recovered DNA was used to transform chemically competent *E. coli* DH5α (Life Technologies, Gaithersburg, Md.) or ElectroMax DH10B (Life Technologies) by electroporation. Electroporation was performed with the BTX Electro Cell Manipulator 600 (Biotechnologies and Experimental Research, Inc., San Diego, Calif.). Recipient cells were subjected to a single 5-ms pulse at a field strength of 5.5 kV/cm using a 2-mm gap Gene Pulser cuvette electrode (Bio-Rad, Hercules, Calif.) at room temperature. After a 1-h recovery period in NZY broth at 37°C, the transformed bacteria were plated onto low-salt Luria-Bertani plates containing either 100 μg of blasticidin per ml or 25 μg of zeocin per ml.

***lac* repressor-mediated recovery of integrated retroviral DNA.** *lac* repressor-mediated recovery was carried out essentially as described previously (16, 17). Either 200 or 100 μg of genomic DNA from the RSVP(A)B-infected DF-1 cells or RSVP(A)Z-infected DF-1 cells, respectively, was digested with *Cla*I. The reaction mixture was adjusted to 150 mM NaCl, 10 mM EDTA, 50 μg of bovine serum albumin per ml, and 10% (vol/vol) glycerol in a final volume of 600 μl. The digested DNA was incubated with 6 μg of *lac* repressor protein (kindly provided by P. Lu) for 30 min at room temperature. The DNA-*lac* repressor protein mixture was then filtered through nitrocellulose which had been pretreated with 0.4 M potassium hydroxide and washed twice with water. The nitrocellulose filter was washed three times with 1.5 ml of wash buffer (150 mM NaCl, 10 mM EDTA) and eluted twice with 1 ml of elution buffer (10 mM Tris-HCl [pH 7.5], 10 mM EDTA, 10 mM isopropyl-β-D-thiogalactopyranoside [IPTG]) for 30 min at 37°C. The enriched DNA was extracted with phenol-chloroform and precipitated with ethanol. The precipitated DNA was ligated with the Rapid DNA

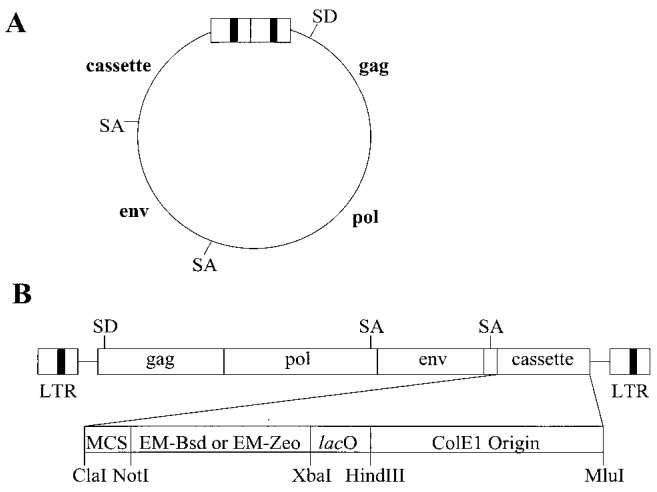

FIG. 1. Schematic drawing showing the structure of the RSVP vector. (A) RSVP. The viral genes *gag*, *pol*, and *env* are shown (not to scale). The positions of the splice donor (SD) and SA are also shown. (B) Schematic representation of the cassette. EM-Bsd, EM-7 promoter-blasticidin resistance gene; EM-Zeo, EM-7 promoter-zeocin resistance gene; *lacO*, *lac* operator.

ligation kit (30 U/200 μl; Roche, Indianapolis, Ind.) for 18 h at 16°C. The ligated DNA was extracted with phenol-chloroform, precipitated with ethanol, and resuspended in 8 μl of water. Two microliters of DNA was used for transformation as described above.

Sequencing of the integrated viral DNA. DNA sequences were determined by cycle sequencing with a primer specific for the U5 region of RCASBP using a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.). Sequencing reactions were analyzed with an automated 373A DNA sequencer (Applied Biosystems). The sequence of the primer was 5′-ACCACATTGGTGTGCACCTGG-3′.

Southern blot. Genomic DNA was isolated from the infected cells using the cell culture DNA Midi kit (Qiagen, Valencia, Calif.) following the manufacturer's recommendations. For Southern blot analyses, 15 μg of genomic DNAs was digested with an appropriate restriction enzyme (*Eco*RI) and separated by electrophoresis on 1% agarose–Tris-acetate-EDTA buffer gels. DNA samples were denatured with alkali, neutralized, and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) by capillary blotting with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Membranes were baked at 80°C under vacuum for 2 h. The hybridization probe (encompassing *env* to the long terminal repeat [LTR] region) was a 1.2-kb *Eco*RI fragment purified from RCASBP(A) and labeled with [α-^{32}P]dCTP using Prime-It II (Stratagene, La Jolla, Calif.). Prehybridizations and hybridizations were carried out overnight at 42°C. Hybridization solution contained 50% formamide, 1× Denhardt solution, 0.5% sodium dodecyl sulfate, 7.5% dextran sulfate, and 5× SSC. After stringent washes, membranes were autoradiographed for 1 day.

RESULTS

Construction of replication-competent RSVP. We constructed RSVP by inserting a cassette (MCS-drug resistance gene-*lac* operator-ColE1) into the replication-competent avian retroviral vector 779BP. 779BP is a derivative of 779/2795 that was constructed by replacing the *pol* region of 779/2795 with the corresponding *pol* region of the Bryan high-titer strain of RSV (18, 23). In terms of the retroviral vector portion carried by the plasmid, 779/2795 is the same as RCAN(A) and 779BP is the same as RCANBP(A). The details of this construction are given in Materials and Methods. The final form of the plasmids is shown in Fig. 1. The cassette contains one of two drug resistance genes, either blasticidin or zeocin resistance. The RSVP vectors contain a splice acceptor (SA) sequence immediately upstream of the cassette. The drug resistance gene is expressed in avian cells as a subgenomic mRNA from the viral promoter within the LTR, whereas in bacteria the same gene is expressed from the EM-7 bacterial promoter. The EM-7 promoter is synthetic and was isolated from the plasmids carrying the blasticidin and zeocin resistance genes (see Materials and Methods). To provide a simple and rapid purification of the vector DNA, the perfectly symmetric *lac* operator sequence was inserted in the cassette. Finally, the cassette contains a bacterial origin of replication (ColE1) to allow closed circular forms of viral DNA to replicate as plasmids in bacteria.

Recovery of unintegrated viral DNA in *E. coli*. To rescue unintegrated retroviral DNA, the RSVP vectors were transfected into DF-1 cells and the transfected cells were passaged two times to let the viruses spread throughout the culture. RSVP(A)Z virus confers resistance to zeocin and the RSVP(A)B virus confers resistance to blasticidin. Because the viruses are replication competent, it was not possible to determine the titer in DF-1 cells. However, the RSVP vectors appear to replicate as efficiently after transfection (or infection) as the corresponding RCASBP(A) vectors. The viral supernatant was harvested and used to infect fresh DF-1 cells. To show that unintegrated viral DNAs present in these infected DF-1 cells were due to the successful viral infection and not because of carryover of RSVP plasmids from the transfected DF-1 cells, the circularized unintegrated retroviral DNAs were obtained from infected cells by the method of Hirt. The DNA was used to transform *E. coli*, selecting either blasticidin- or zeocin-resistant colonies. To demonstrate that the viral DNAs rescued from infected cells contained both of the circular forms of viral DNA (one-LTR and two-LTR circles), restriction digests were carried out. The starting plasmid, used to initiate infection, was a two-LTR circle. The only *Sac*I site in RSVP is located between the LTR and the *gag* gene, and the *Cla*I site is between *env* and the LTR. Thus, digestion with *Cla*I and *Sac*I should yield two fragments, a 6.8-kb vector DNA fragment and either a 2.1-kb fragment if the proviral DNA contains two LTRs or a 1.8-kb fragment if it contains one LTR (Fig. 2A). For RSVP(A)B, of the 13 recovered plasmids, 4 contained only one LTR, 2 contained two LTRs (Fig. 2B), and 7 were the products of autointegration events (data not shown) (15, 22). Similarly, for RSVP(A)Z, of the 20 recovered plasmids, 10 contained one LTR, 3 contained two LTRs (Fig. 2C), and 7 were autointegration products (data not shown). This result suggests that proviral DNAs observed in infected cells were due to successful replication of RSVP RNA and transfer of viral particles and not due to carryover of RSVP from the initial transfection.

Although we were able to get appropriate clones, the recovery was relatively low with chemically competent cells. We tried using electroporation to increase the transformation efficiency (see Materials and Methods for details). When the same amount of DNA sample was used to transform *E. coli* by electroporation, we obtained 238 and 802 transformants from RSVP(A)B and RSVP(A)Z, respectively. These results suggest that the efficiency of unintegrated plasmid recovery was 15 to 40 times higher with electroporation.

Rescue of integrated viral DNA. To rescue integrated viral DNA, genomic DNA was isolated from infected cells and digested with *Cla*I. Since one *Cla*I site comes from the RSVP

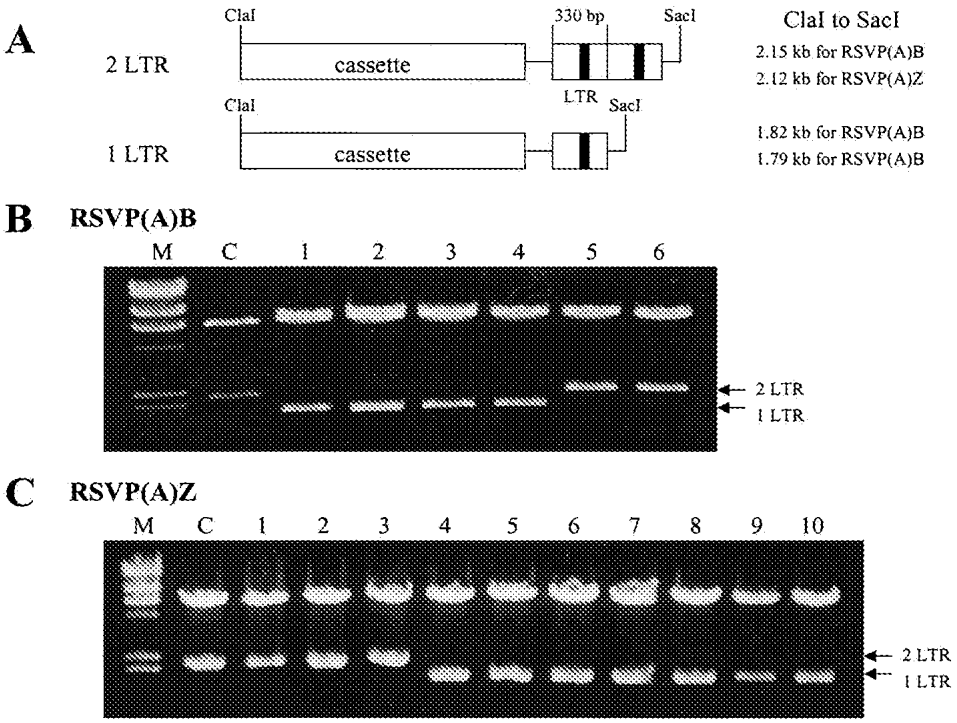

FIG. 2. Rescue of unintegrated retroviral DNA. Low-molecular-weight DNA was isolated by HIRT extraction and the extracted DNA was used to transform *E. coli* DH5α. The selection was for either blasticidin- or zeocin-resistant colonies. (A) Diagram showing the difference between two-LTR and one-LTR DNA segments. (B and C) Plasmids were digested with *Cla*I and *Sac*I. The positions of DNAs containing two LTRs and one LTR are indicated. C, parental RSVP vector; M, size marker (λ DNA digested with *Hin*dIII).

vector, the second *Cla*I site must come from the adjacent cellular DNA (Fig. 3A). The *Cla*I-digested DNAs were enriched for viral sequences by binding to the *lac* repressor protein (see Materials and Methods) and then self-ligated. These enriched fractions were used to transform *E. coli*. Restriction cleavage analyses with *Cla*I and *Mlu*I showed that 3 of the 16 RSVP(A)B transformants and 3 of the 7 RSVP(A)Z transformants contained cellular DNA (Fig. 3B). For these plasmids, double digestion with *Cla*I and *Mlu*I generated a band representing the cassette insert. In addition, the digestion yielded

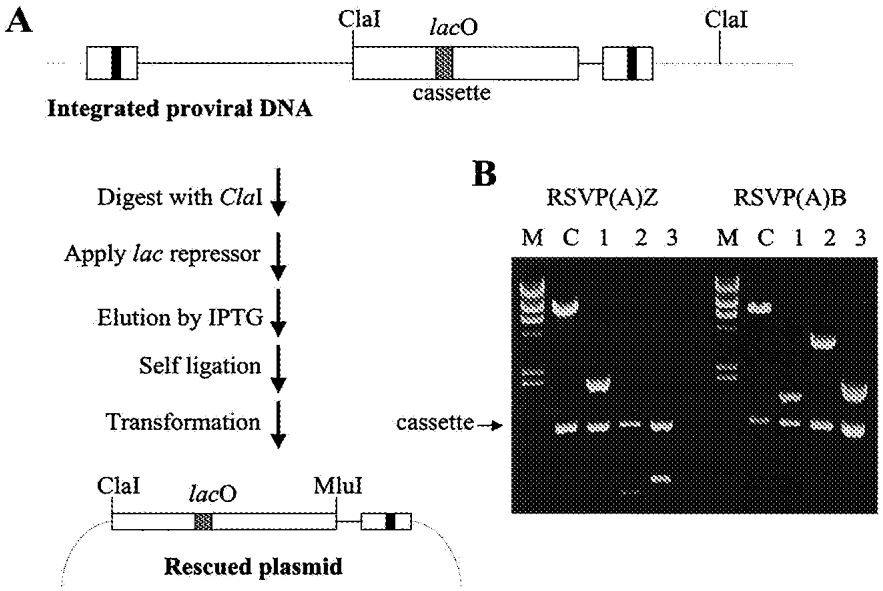

FIG. 3. Rescue of integrated retroviral DNA. (A) Schematic diagram of *lac* repressor-mediated recovery (see Materials and Methods for details). (B) Plasmids were digested with *Cla*I and *Mlu*I. The position of the cassette insert is indicated. C, parental RSVP vector; M, size marker (λ DNA digested with *Hin*dIII).

	LTR-U5 ▼ LTR-U3
RSVP	...AAGGCTTCATTAATGTAGTCTT...
RSVP(A)B #1	...AAGGCTTCA**TTTGGTGACCAGA**...
RSVP(A)B #2	...AAGGCTTCA**GACAGGTGTAGTT**...
RSVP(A)B #3	...AAGGCTTCA**AGAAGATTCAGAA**...
RSVP(A)Z #1	...AAGGCTTCA**GCCTTGATTTATT**...
RSVP(A)Z #2	...AAGGCTTCA**TTAAGACAGCTAT**...
RSVP(A)Z #3	...AAGGCTTCA**CCATTTTTAAGAT**...

FIG. 4. Sequences of integrated retroviral DNA. The inverted triangle indicates the boundary between the two LTRs. Sequences flanking the LTR are shown in bold.

another band of variable size due to the variable position of the nearest *Cla*I cleavage site in the cellular DNA. To demonstrate whether these inserts were derived from the cellular DNA, rescued plasmids were directly sequenced with a primer derived from the U5 sequence of the viral vector. As expected, all plasmids showed the integration-specific sequence feature that a CA dinucleotide pair was present at the site of joining to host DNA (Fig. 4). RSVP(A)B clones 2 and 3 and RSVP(A)Z clones 1 and 3 appear to contain novel inserts derived from the cellular DNA. However, sequencing results revealed that RSVP(A)B clone 1 and RSVP(A)Z clone 2 contained inserts identical to part of the RSVP vector sequence, indicative of autointegration (22).

As described above, we tried electroporation to obtain additional integration sites. When the same amount of enriched DNA sample was used to transform *E. coli* by electroporation, we were able to get 1,760 and 886 transformants from RSVP (A)B and RSVP(A)Z, respectively; thus, the efficiency of enriched plasmid recovery was more than 100 times higher with electroporation.

When we recovered integrated viral DNA, in addition to the plasmids containing integration sites we also obtained plasmids of approximately 3 kb in more than half of the clones. Further analyses of these plasmids revealed that they were by-products derived from the circularized unintegrated viral DNAs. There is a second *Cla*I site within the *gag* gene (Fig. 5A). In plasmids grown in *E. coli,* this site is subject to dam methylation. However, there is no dam methylation in avian cells. When genomic DNA isolated from the infected DF-1 cells was digested with *Cla*I, both *Cla*I sites in the circular unintegrated viral DNAs were cleaved and the cassette (*lacO*)-containing DNA segment was enriched by binding to the *lac* repressor protein. These 3-kb plasmids were generated by self-ligation and were recovered by transformation into *E. coli.* After transformation and growth in *E. coli*, the *Cla*I site is again subject to dam methylation and thus digestion with *Cla*I did not cut the 3-kb mini plasmids. Digestion with *Mlu*I cut the mini plasmids once and digestion with *Bam*HI generated two fragments, of 1.54 and 1.39 kb, as expected (Fig. 5B). The recovery of the 3-kb mini plasmid provides additional evidence that the unintegrated viral DNAs were generated by successful viral infection rather than carryover of initial RSVP plasmids from the transfected DF-1 cells.

Stability of vectors. To determine that the cassette insert is reasonably stable upon viral passage in avian cells, genomic DNAs derived from virus that had been passaged under non-

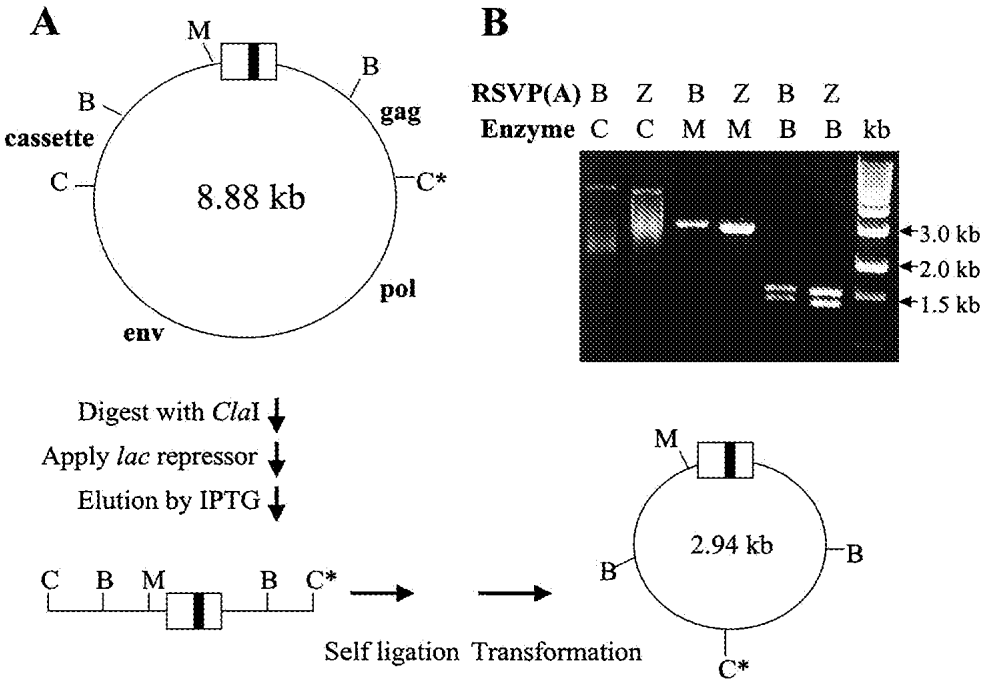

FIG. 5. Major by-product obtained in the recovery of integrated viral DNA. (A) Schematic drawing of the generation of the plasmid. M, *Mlu*I; B, *Bam*HI; C, *Cla*I; C*, *Cla*I site within *gag* gene subjected to dam methylation in *E. coli* but not in avian cells. (B) Restriction enzyme analyses of the plasmid. Digestion with *Cla*I did not cut the plasmid due to dam methylation. Digestion with *Mlu*I cut the plasmid once and digestion with *Bam*HI generated two fragments, of 1.54 and 1.39 kb, as expected. kb, DNA ladder in kilobases.

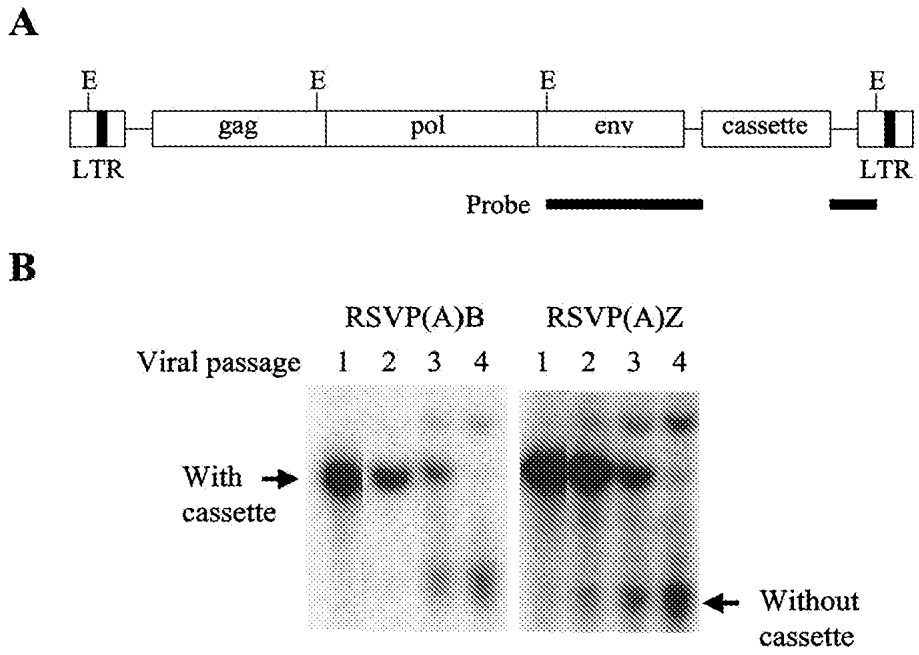

FIG. 6. Southern transfer analyses of the stability of the RSVP vector. (A) The probe was prepared from RCASBP(A) as a 1.2-kb *Eco*RI fragment to provide equal opportunity to hybridize to *Eco*RI fragments that contained (or had lost) the cassette. *Eco*RI recognition sites are indicated (E) (not to scale). (B) Detection of the cassette insert in the genomic DNA derived from cells infected with the RSVP(A)B and RSVP(A)Z vectors. Genomic DNA was digested with *Eco*RI, resolved in an agarose gel, transferred onto a nitrocellulose membrane, and hybridized with ^{32}P-labeled DNA prepared from the 1.2-kb *Eco*RI fragment of RCASBP(A). The larger band represents a 2.4-kb *Eco*RI fragment containing the insert, whereas the smaller band represent a 1.2-kb *Eco*RI fragment lacking the insert.

selective conditions were digested with the restriction enzyme *Eco*RI and analyzed by Southern blot. To provide equivalent hybridization to *Eco*RI fragments that contained the cassette and the related fragments from viral genomes that had lost the cassette, the probe was prepared from RCASBP(A) as a 1.2-kb *Eco*RI gel-purified DNA fragment (Fig. 6A). As can be seen in Fig. 6B, the cassette insert was present in the vector up to three viral passage for both drugs, but there was significant loss in the fourth viral passage, suggesting that RSVP vectors are stable for approximately three viral passages. The efficiency of transformation does affect the apparent stability of the insert; a relatively efficient transformation gives a larger burst of virus and fewer rounds of replication are required to get the virus stock. With this caveat, the stability of the two drug cassettes are similar to an "average" insert in RCASBP(A).

DISCUSSION

We have developed two related replication-competent retroviral shuttle vectors (RSVPs). The sequences that permit the viral DNA to replicate as a plasmid and to be selected in *E. coli* (either blasticidin resistance or zeocin resistance) are small (approximately 1.2 kb), which should make it possible to insert additional sequence in these vectors. The drug resistance genes were inserted in a fashion that permits expression of the selectable markers (via a spliced message) in avian cells; as expected, infection with the vectors confers resistance. The inserts are relatively stable during viral passage; no appreciable loss of the inserts was seen until the fourth viral passage.

One of the problems with recovering shuttle vector DNA after infection is the large amount of contaminating cellular DNA. Unintegrated viral DNA is, in general, easier to recover and clone than integrated DNA, presumably because it is possible to use physical fractionation to enrich for the closed circular forms of unintegrated viral DNA.

We particularly wanted to develop a vector system that makes it simple to recover both unintegrated and integrated DNA. To simplify enrichment of viral DNA, we included the *lacO* sequence in the inserted segment. DNAs containing the *lacO* sequence can be rapidly enriched by binding to *lac* repressor protein (16, 17). The DNA-protein complex is captured on a nitrocellulose filter, and then the DNA is selectively eluted by the addition of IPTG. This simple protocol allowed us to directly clone integration sites. In the initial experiments we used chemically competent cells and were able to get valid clones. However, the cloning of unintegrated viral DNA was more-than-100-fold more efficient when the DNA was introduced into *E. coli* by electroporation.

In addition to recovering integration sites, we want to use these vectors to aid in the recovery of viral vectors that have been adapted by passage. In previous experiments we have found that, in some cases, the initial version of a vector replicated poorly. In some cases, it is possible, with a replication-competent virus, to adapt the vector by passage (1). Variant viruses that grow better have a selective advantage. The adapted virus is obtained simply by repeated passage of the viral stock either in cultured cells or in infected embryos. However, to be used as a vector, the adapted virus must be molecularly cloned and characterized. A shuttle vector should be quite useful in adaptation protocols, provided that the insert is stable enough to be retained during the adaptation process.

The zeocin and blasticidin inserts are stable for three viral passages in the RSVP vectors, even in the absence of drug selection, and should be useful for recovering adapted viruses.

The plasmid cassettes are small and are flanked by *Cla*I sites. It should be possible to introduce these prokaryotic cassettes into other viral vectors. In cases in which the vector will not generate an appropriately spliced message for the selectable markers, the cassette could be linked to an internal promoter or an internal ribosome entry site.

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